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ORIGINAL REPORT

Identification of Leptomeningeal Disease in Aggressive B-Cell Non-Hodgkin's Lymphoma: Improved Sensitivity of Flow Cytometry

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ABSTRACT

Purpose
Here, we evaluate the sensitivity and specificity of a new 11-parameter flow cytometry (FCM) approach versus conventional cytology (CC) for detecting neoplastic cells in stabilized CSF samples from newly diagnosed aggressive B-cell non-Hodgkin's lymphoma (B-NHL) at high risk of CNS relapse, using a prospective, multicentric study design.

Patients and Methods
Moreover, we compared the distribution of different subpopulations of CSF leukocytes and the clinico-biologic characteristics of CSF+ versus CSF−, patients, in an attempt to define new algorithms useful for predicting CNS disease.

Results
Overall, 27 (22%) of 123 patients showed infiltration by FCM, while CC was positive in only seven patients (6%), with three other cases being suspicious (2%). CC+/FCM+ samples typically had more than 20% neoplastic B cells and/or ≥ one neoplastic B cell/μL, while FCM+/CC− samples showed lower levels ($P < .0001$) of infiltration. Interestingly, in Burkitt lymphoma, presence of CNS disease by FCM could be predicted with a high specificity when increased serum β2-microglobulin and neurological symptoms coexisted, while peripheral blood involvement was the only independent parameter associated with CNS disease in diffuse large B-cell lymphoma, with low predictive value.

Conclusion
FCM significantly improves the sensitivity of CC for the identification of leptomeningeal disease in aggressive B-NHL at higher risk of CNS disease, particularly in paucicellular samples.

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INTRODUCTION

Involvement of the CNS is a relatively uncommon[1-4] and adverse clinical complication of non-Hodgkin's lymphoma (NHL).[1,3-5] The variable frequency of leptomeningeal disease reported so far in B-cell (B-) NHL[1,5-8] may be explained by the different histologic subtypes of B-NHL analyzed, the criteria used to define CNS infiltration, the inclusion of newly diagnosed versus recurrent patients, and/or the use of CNS prophylaxis.[2,5,6-10] B-NHL features which have been associated with CNS involvement include high histopathologic grade, aggressive B-cell neoplasias (eg, Burkitt's lymphoma [BL]), presence of testicular, bone marrow (BM), or Waldeyer ring involvement, retroperitoneal bulky mass (> 10 cm), and increased serum lactate hydrogenase (LDH) with an estimated CNS infiltration rate at diagnosis of around 1% to 21%.[1,2,7,8,10-12] In contrast, CNS infiltration in low-grade lymphomas is considerably lower (< 5% of the patients).[5] Based on the aforementioned criteria, B-NHL patients are currently selected for CNS prophylaxis.[10] However, the predictive value for CNS disease of these parameters remains limited with both a relatively low sensitivity and specificity.[5,6,13]

Currently, diagnosis of CNS disease is frequently suspected on presence of clinical manifestations of CNS involvement and neuroimaging techniques,[1,14] and it is confirmed by conventional cytomorphological (CC) analysis of CSF.[6,15-17] While highly specific, CC is associated with a limited sensitivity with up to 20% to 60% false-negative results.[4,9,18] In addition, interpretation of

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cytological findings may be difficult because of paucity of cells in CSF and the morphological similarities between benign and malignant cells.[9,16,17,19,20]

Recently, evaluation of complementary diagnostic tools to the study of CSF (eg, immunophenotypic, molecular, and cytogenetic techniques)[17,20] has shown that flow cytometry (FCM) could be more sensitive than CC for the detection of CSF involvement by mature neoplastic lymphoid cells,[4,9,20-22] supporting the combined use of the two methods in both newly diagnosed and relapsed/treated B-NHL.[4,9,20,22,23] However, most CSF samples are paucicellular and contain a limited number of cells with a rapidly decreasing viability,[20] which have hampered the application of FCM in prospective, multicentric trials.[20,24]

In this study, we prospectively evaluated the sensitivity and specificity of a standardized 11-parameter FCM immunophenotypic approach versus CC for detecting neoplastic cells in stabilized CSF samples from newly diagnosed aggressive B-NHL at high risk of CNS relapse; moreover, we compared the disease characteristics of aggressive B-NHL with positive versus negative CSF samples in an attempt to define new algorithms that could predict for CNS disease. Our results show a significantly higher sensitivity for FCM and provide new algorithms for identifying BL patients, at very high risk of CNS disease.

PATIENTS AND METHODS

Patients and CSF Samples

Since March 2006, a total of 123 CSF samples were obtained after informed consent was given, from an identical number of newly diagnosed patients: 78 males and 45 females, mean age of 55 ± 18 years, ranging from 13 to 92 years, with aggressive B-NHL, considered to be at high risk for CNS disease, and recruited at 29 different hospitals in Spain. Diagnosis of B-cell NHL was established according to the WHO criteria[25] with the following distribution: diffuse large B-cell lymphoma (DLBCL), 81 patients; BL, 31 patients; follicular lymphoma transformed to DLBCL (tFL), four patients; T-cell rich B-NHL, three patients; mediastinal DLBCL, one patient; intravascular lymphoma, one patient; and plasmablastic lymphoma, two patients. The study protocol was approved by the local ethics committee of individual participating centers. Inclusion criteria were: aggressive B-NHL patients with either infiltration of extranodal sites, including testis, breast, paranasal sinus, and/or BM, neurological symptoms, and/or increased serum LDH levels; definition of BM involvement required the presence of an aggressive B-NHL. The most relevant clinical and biologic characteristics of the patients analyzed (median follow-up of 11 months; range, 3 to 22 months) are summarized in Table 1.[26] From those BL patients showing increased β2-microglobulin levels (67%), all but two patients had normal serum creatinine levels (< 1.2 mg/dL). In all patients, CSF samples were analyzed in parallel at the institution of origin using CC, in combination with immunocytochemistry (CD20 and CD10 and/or Ig light chains), in cases suspected of CSF infiltration, and centrally at the Cytometry Service of the University of Salamanca (Salamanca, Spain) using multiparameter FCM. Results obtained by CC were reported as negative, suspicious, or positive for malignant cells and all discrepant cases were reviewed by another experienced laboratory hematologist/cytologist.

FCM Immunophenotyping and Other Complementary Studies

For multiparameter FCM analyses, CSF samples (median volume, 2.0 mL; range, 0.5 to 4.0 mL) were directly collected into tubes containing EDTA and 0.2 mL of Transfix (Immunostep SL, Salamanca, Spain) and shipped overnight to the central FCM laboratory. Immediately on arrival at the central laboratory, the volume of the CSF sample was measured (after subtracting 0.2 mL corresponding to the Transfix solution) and recorded; then, 2 mL of sterile/filtered phosphate buffered saline ([PBS]; pH, 7.4) was added to the sample and the sample was centrifuged (5 minutes at 540 g), the supernatant discarded and the cell pellet resuspended in 300 μL of filtered PBS. Afterward, 100 μL of the concentrated cell suspension was stained for 15 minutes at room temperature in the darkness, with the following six-color combination—fluorescein isothiocyanate/phycoerythrin/peridinin clorophyll protein-cyanin 5.5/phycoerythrin-Cy7/allophycocyanin (APC)/APC-CY7- of 9 antibodies (MAb): CD8-sIgλ/CD56-sIgκ/CD4-CD19/CD3/CD20/CD45. After staining, 2 mL of FACS lysing solution (Becton/Dickinson Biosciences, San José, CA) was added. After 5 minutes incubation (room temperature), samples were sequentially centrifuged (5 minutes at 540 g) and resuspended in 100 μL of premixed Perfect-COUNT microspheres (Cytognos SL, Salamanca, Spain), immediately before data acquisition. After gentle mixing, samples were run on a FACSCanto II flow cytometer (Becton/Dickinson Biosciences) equipped with the FACSDiva software (Becton/Dickinson Biosciences) and information about all events corresponding to nucleated cells present in the stained sample aliquot was acquired. For data analysis, the INFINICYT software (Cytognos SL) was used. Absolute cell numbers per unit of sample volume were calculated using the following formula:

$$\text{No. of Perfect COUNT beads}/\mu\text{L added} \times \%\ \text{of cells}/\%\ \text{of beads} \times \text{CSF sample volume}\ (\mu\text{L})$$

Normal and neoplastic leukocyte populations present in each sample were identified through a boolean gating strategy based on forward scatter, side scatter, and CD45 expression (Fig 1A and 1B). Identification of B cells was based on the expression of CD19 and/or CD20 in the absence of CD3 (Fig 1K and 1I). Both surface immunoglobulin (slg) light chain restriction and/or the presence of an abnormal forward scatter/side scatter/CD19/CD20 phenotypic pattern were used to establish the presence of monoclonal versus polyclonal B-cell populations. CD4+ and CD8+ T-cells were defined as being CD3+/CD4+ or CD3+/CD8+. Natural killer cells were identified as those CD45high/CD56+/CD3− events showing low scatter characteristics, while monocytic cells and neutrophils were defined on the basis of their relatively higher light scatter properties, their unique pattern of CD45 expression and, in case of monocytes, dim CD4 expression. Finally, plasma cells were identified based on their heterogeneous CD45 expression and positivity for CD19 in the absence of sIg. In order to increase the sensitivity of the assay, if no CSF infiltration by neoplastic cells was detected, the remaining centrifuged sample (200 μL) was restained with an identical combination of MAb also containing CD14-APC. In turn, when a pathological B-cell population was identified in the initial FCM analysis, a complete phenotypic characterization was attempted as previously described.[27]

The overall number of cells analyzed by FCM was of 46 ± 437 cells/μL, (range, 0.02 to 4,852 cells/μL). In nine of 27 FCM+ patients with high cell counts, fluorescence in situ hybridization (FISH) and FCM DNA ploidy studies were performed[28,29]: 538 ± 1,517 CSF cells/μL containing 518 ± 1,475 neoplastic cells/μL (range, 1 to 4,852 cells/μL and 0.1 to 4,712 cells/μL, respectively). In contrast, CSF samples from other FCM+ patients showing low CSF cell counts −3 ± 5 CSF cells/μL containing 0.5 ± 1.4 neoplastic cells/μL (range, 0.02 to 18 cells/μL and 0.003 to 6 cells/μL, respectively) were not available for FISH and DNA studies. The minimal number of clonally restricted and/or phenotypically aberrant B cells to define CSF infiltration by FCM was of 10 clustered events.

Statistical Analyses

In order to establish the statistical significance of differences observed between groups, either the χ^2 or the Mann-Whitney U tests were used for categoric and continuous variables, respectively. Logistic regression analysis was separately applied for the identification of the best combination of clinical and biologic parameters associated with CSF involvement in DLBCL and BL patients. $P \leq .05$ was considered to be associated with statistical significance.

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Table 1. Clinical and Laboratory Characteristics of Aggressive B-NHL Patients Distributed According to the Presence Versus Absence of Leptomeningeal Disease as Detected by Multiparameter FCM Immunophenotyping (n = 123)

	Cases by FCM (%)									
	CNS+ (n = 27)			CNS− (n = 96)				Total Cases (n = 123)		
Patient Characteristic	%	Mean	SD	%	Mean	SD	*P*	%	Mean	SD
Age, years*		59	20		54	18	NS		55	18
Range		18-92			13-84				13-92	
Sex							NS			
Male	63			63				63		
Female	37			37				37		
Performance status (ECOG ≥ 2)	**67**			**38**			**.02**	**43**		
B symptoms	58			44			NS	45		
Neurological symptoms	**57**			**10**			**< .0001**	**20**		
Adenopathies	78			72			NS	71		
Hepatomegaly	22			18			NS	18		
Splenomegaly	22			24			NS	23		
Histolopathologic diagnosis										
DLBCL	**44**			**72**				**66**		
BL	**44**			**20**			**.01**	**25**		
tFL	**12**			**1**				**3**		
Other B-NHL	**0**			**7**				**6**		
PB infiltration	22			11			.07	11		
BM infiltration	**62**			**31**			**.04**	**35**		
Extranodal tumor	**100**			**80**			**.03**	**83**		
Infiltration of ≥ 2 extranodal sites	56			34			.08	38		
Stage III/IV	95			72			.07	72		
Standard IPI (No. of IPI factors)										
Low (0, 1)	23			23				23		
Low intermediate (2)	19			22			.07	21		
High intermediate (3)	11			32				28		
High (4, 5)	47			23				28		
Revised IPI[26]										
Very good (0)	4			11				10		
Good (1, 2)	38			32			NS	34		
Poor (3, 4, 5)	58			57				56		
HIV positive	9			13			NS	12		
Laboratory parameters*										
WBC count, × 10^9/L		7.3	3		7.7	4	NS		7.6	4
Range		1.1-14			0.8-27				1-27	
Lymphocyte count, × 10^9/L		1.5	1.2		2	2.2	NS		2	2.1
Range		0.3-6			0.2-18				0.2-18	
Hemoglobin, g/L		11	2		11	2	.02		11	2
Range		7-15			6-16				6-16	
Platelet count, × 10^9/L		248	169		270	143	NS		265	148
Range		25-736			19-780				20-780	
Serum LDH, U/L		2,166	3,622		1,741	6,180	NS		1,830	5,728
Range		200-15,000			154-55,550				154-55,550	
Serum β2-microglobulin, mg/dL		**4.2**	**3**		**3**	**2.5**	**.04**		**3.2**	**3**
Range		**0.9-13**			**0.1-14**				**0.1-14**	
Total serum proteins, g/dL		6	1		6.4	1	NS		6	1
Range		4.2-10			4.1-9				4.1-10	
Total CSF proteins, mg/dL		65	69		43	21	NS		46	34
Range		9-259			17-120				9-258	
CSF volume, mL		2.1	0.7		2	0.7	NS		2	0.7
Range		1-4			0.5-4				0.5-4	
CSF cell count/uL		**201**	**930**		**2**	**4**			**46**	**437**
Range		**0.02-4,852**			**0.01-40.5**		**.001**		**0.01-4,852**	

NOTE. *P* values provided correspond to comparisons between CNS+ and CNS− aggressive B-NHL patients by multiparameter FCM. Bold font highlights variables for which statistically significant differences were found between CSF+ compared with CSF− patients.

Abbreviations: B-NHL, B-cell non-Hodgkin's lymphoma; FCM, flow cytometry; SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; IPI, International Prognostic Index; DLBCL, diffuse large B-cell lymphoma; tFL, follicular lymphoma transformed to DLBCL; BL, Burkitt's lymphoma; BM, bone marrow; PB, peripheral blood; LDH, lactic dehydrogenase; NS, no statistically significant differences found ($P > .05$).

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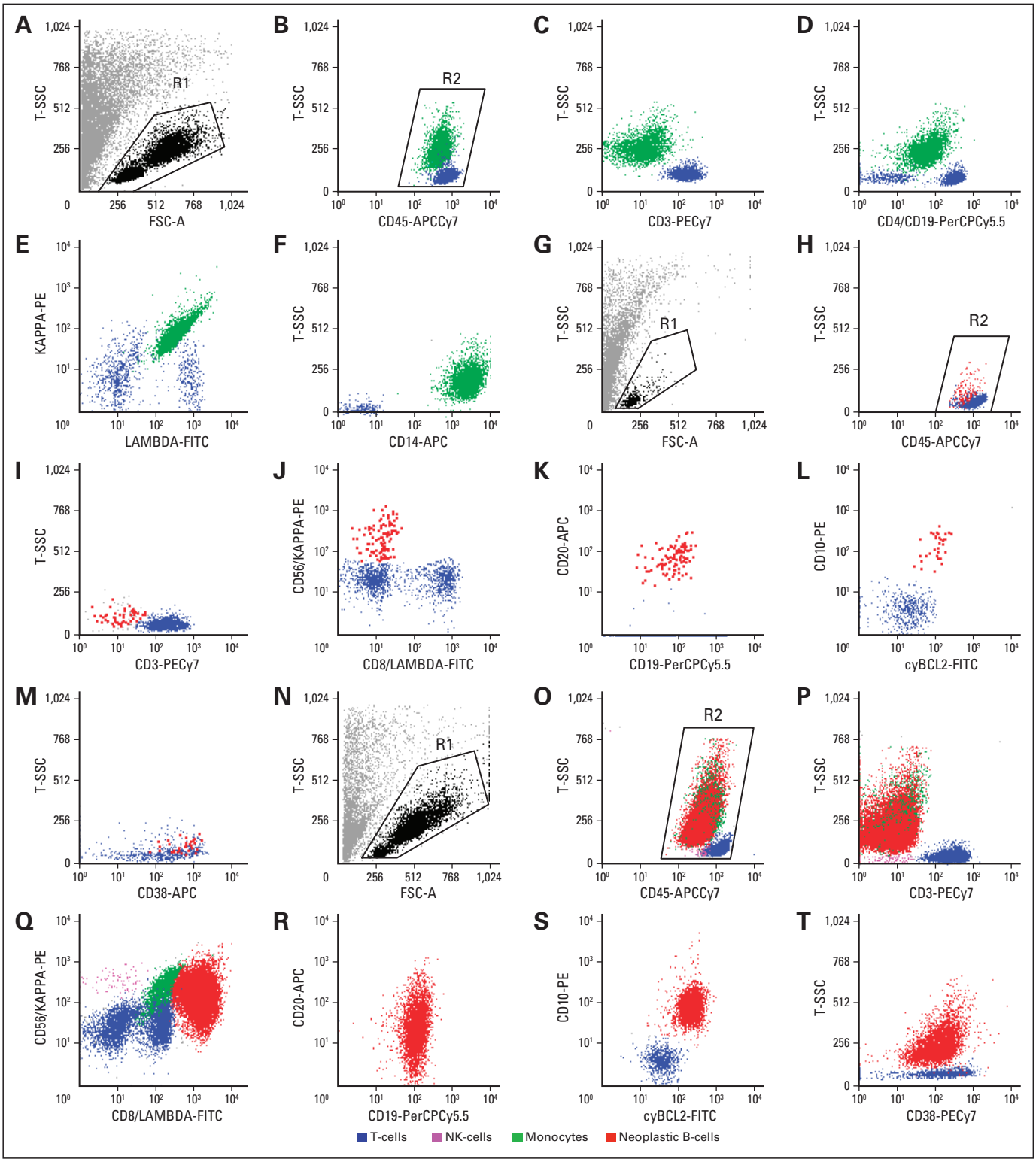

Fig 1. Illustrating bivariate dot plot histograms of (A-F) a normal/reactive CSF sample from a diffuse large B-cell lymphoma (DLBCL) patient showing no CSF infiltration by both multiparameter flow cytometry (FCM) immunophenotyping and conventional cytology (CC) in comparison to two CSF samples from two different patients showing (G-M) low and (N-T) high levels of CSF disease, as detected by multiparameter FCM immunophenotyping. In all bivariate dot plots except those in (A,G, N), nonleukocyte events (gray dots) were excluded by gating on forward scatter (FSC) versus side scatter (SSC; R1 region in A,G, N) and SSC versus CD45 antigen expression (R2 region in B, H, O). In the other panels, the distribution in the three different samples of the distinct cell subsets identified, including T lymphocytes (CD45+, CD3+, CD8−, and CD8+; blue dots), natural killer cells (CD56+, CD3−, CD45high; violet dots) and neoplastic B cells (CD45+, sIg+, CD10+, Bcl2high, CD38++, CD19+, and CD20+; red dots), are displayed.

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Table 2. Detection of CNS Disease in Aggressive B-NHL: Results Obtained Using Multiparameter Flow Cytometry Immunophenotyping Versus Conventional Cytology (n = 123)

Conventional Cytology	Flow Cytometry			
	Negative		Positive	
	No.	%	No.	%
Negative	95/123	77	17/123	14
Positive	1/123*	1	7/123	6
Suspicious	—		3/123	2

*The presence of neoplastic cells in this patient was ruled out by further immunocytochemical analyses (one cytospin slide was fixed in acetone and stained with CD20 monoclonal antibody L-26 [Dako, Glostrup, Denmark] using the ABC method).

RESULTS

Frequency of Leptomeningeal Disease As Identified by Multiparameter FCM Versus CC

Overall, multiparameter FCM showed the presence of neoplastic B cells in 27 (22%) of 123 aggressive B-NHL, while CC was either positive or suspicious in only seven (6%) and three (2%) of these patients ($P < .001$), respectively. All except one of the CC+ patients, also showed neoplastic B cells by FCM, the discrepant case corresponding to a false-positive CC result (Table 2). Interestingly, both the absolute count and the percentage of neoplastic B cells identified by FCM were higher ($P < .0001$) in patients in whom both methods were positive, with respect to FCM+/CC− CSF samples (Fig 2). Interestingly, a clear cutoff could be established among FCM+ patients between FCM+/CC− and FCM+/CC+ plus suspicious patients, the later showing CSF infiltration levels typically higher than 20% and ≥ 1 neoplastic B cell/μL. Examples of representative FCM−/CC−, FCM+/CC−, and FCM+/CC+ CSF samples are shown in Figure 1. Detailed immunophenotypic and genetic features of CSF neoplastic B cells from all FCM+ CSF samples, are summarized in Appendix Table A1 (online only).

Genetic Analysis of CSF Infiltrated Samples

In nine of 27 FCM+ patients, FISH confirmed the presence of t(14;18) in two tFL patients and of t(8;14) in five of five BL and one of two tFL analyzed patients (Table A1). Interestingly, in one of three tFL patients studied, neoplastic B cells showed coexistence of t(14;18), *BCL6*, and *c-MYC* gene rearrangements (Table A1). FCM DNA ploidy studies were performed in eight of nine patients, revealing presence of DNA hyperdiploidy in three patients (two BL and one tFL; Table A1).

Distribution of Different Subpopulations of Leukocytes in FCM+ Versus FCM− CSF Samples

Overall, no statistically significant differences were found between FCM− and FCM+ CSF samples as regards the relative distribution of the different subsets of normal residual CSF leukocytes, except for a higher ($P < .001$) percentage of monocytes found among CSF− samples (Appendix Fig A1, online only). By contrast, the absolute number of total CD3+ T-cells ($P = .001$), including both the number of CD4+ ($P < .0001$) and CD8+ T cells ($P = .007$), and natural killer cells ($P = .02$) was higher in patients with positive versus negative CSF samples (Fig A1). Normal values are presented in Appendix Table A2 (online only).

Clinical and Biologic Characteristics of Patients With Positive Versus Negative CSF Samples

The frequency of CSF-positive samples was significantly ($P = .01$) higher among BL (12 of 31; 39%) and tFL (three of four patients) versus DLBCL patients (12 of 81; 15%; Table 1). The frequency of patients with neurological symptoms ($P < .0001$), worse performance status (Eastern Cooperative Oncology Group ≥ 2; $P = .02$), extranodal ($P = .03$) and BM involvement ($P = .04$), as well as β2-microglobulin serum levels ($P = .04$) was also significantly higher among FCM+ versus FCM− patients (Table 1). In contrast, similar frequencies of HIV-positive patients, organomegalies, International Prognostic Index, and serum LDH levels were found in FCM+ versus FCM− patients (Table 1). Among patients with neurological symptoms, FCM was more frequently positive than

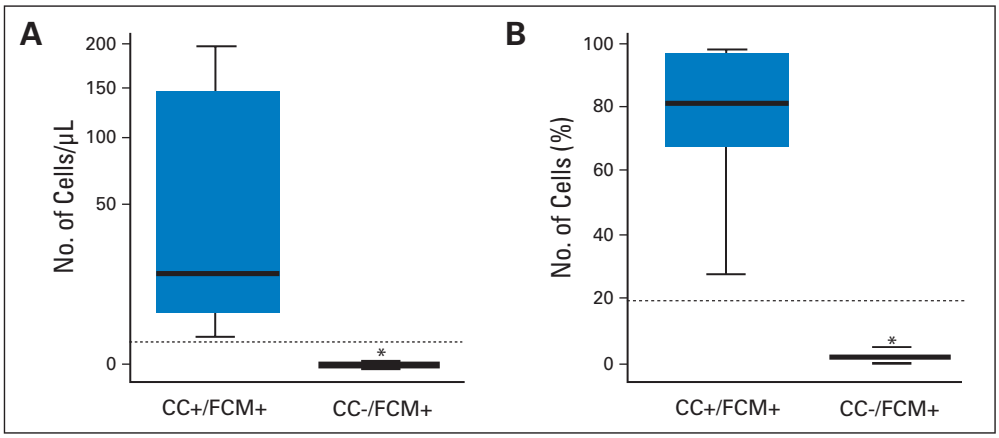

Fig 2. Comparative analysis of the (A) absolute and (B) relative numbers of neoplastic B cells in CSF samples being identified as containing neoplastic B cells by multiparameter flow cytometry (FCM) immunophenotyping, grouped according to the results of conventional cytology (CC). Absolute and relative counts of neoplastic B cells detected in FCM+/CC+ versus FCM+/CC− CSF samples were of 716 ± 1,763 neoplastic B cells/μL (range, 0.9 to 4,712 cells/μL) and of 75% ± 23% neoplastic B cells (range, 28% to 99%) versus 0.06 ± 0.1 neoplastic B cells/μL (range, 0.001 to 0.5 cells/μL) and of 5% ± 8% neoplastic B cells (range, 0.1% to 23%), respectively. Boxes extend from the 25th to the 75th percentiles; the line in the middle and vertical lines represent median values and 95% CIs, respectively. (*) $P < .05$ for comparisons of both absolute and relative numbers in cases showing a positive versus negative CC result.

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Table 3. Detection of CNS Disease in Aggressive B-NHL Patients With Neurological Symptoms (n = 25): Results of Multiparameter Flow Cytometry Versus Conventional Cytology

Conventional Cytology	Flow Cytometry			
	Negative		Positive	
	No.	%	No.	%
Negative	10/25*	40	9/25	36
Positive	—		3/25	12
Suspicious	—		3/25	12

*Only one case showed cranial nerve dysfunction.

CC, ($P < .0001$; Table 3), FCM+ cases also showing a higher frequency of neurological symptoms related with cranial nerve dysfunction (29% *v* 2.1%; $P = .09$).

Multivariate analysis showed that involvement of peripheral blood (PB) was the only independent parameter displaying (low) predictive value for detecting CNS involvement in DLBCL ($P = .05$; Table 4); in contrast, the best combination of independent parameters for CNS involvement in BL included the presence of neurological symptoms ($P = .001$) together with increased serum β2-microglobulin ($P = .001$). The use of a scoring system based on these two parameters, for BL, showed that coexistence of increased serum β2-microglobulin and neurological symptoms (score 2) was highly predictive for CNS disease (Table 4). Despite the significant differences observed in the frequency of neurological symptoms and CSF cell counts in FCM+ versus FCM− patients, these two parameters were associated between them and their combination did not improved discrimination between both groups of CSF samples among DLBCL or BL patients.

DISCUSSION

Herein we report on the largest series of newly diagnosed aggressive B-NHL at relatively high risk of CNS disease, in which infiltration of CSF samples was evaluated in parallel by both FCM and CC, using a prospective multicentric study design. Overall, we show that FCM is by far more sensitive than CC for the detection of CSF involvement in these patients, confirming and extending on previous observations in aggressive B-NHL and other malignant disorders.[4,9,20-23,30,31] As an example, Hegde et al[9] have shown CSF involvement by FCM in 11 of 51 B-NHL analyzed, while CC was only able to detect malignant cells in one of these 11 patients. More recently, Bromberg et al[4] also showed that FCM could detected CSF disease considerably more frequently than CC, the first CSF sample analyzed being already positive by FCM in 73% of the infiltrated patients versus 32% by CC. We demonstrated that such increased rate of FCM+ CSF samples with respect to CC is due to a higher sensitivity of the former technique, since CC was typically positive/suspicious in cases having pleocytosis[4,9,17,20-23,31] with a cutoff level for positivity of higher than 20% neoplastic B cells and/or $\geq$ 1 cell/μL, while FCM was better suited for paucicellular samples.[4,9,17,20] Due to the relatively short follow-up, the clinical significance of a FCM+/CC− result still remains to be determinate. However, it should be noted that more than half of the FCM+/CC− patients showed neurological symptoms, frequently reflecting cranial nerve dysfunction. In addition, Hegde et al have shown recurrence of CNS disease in five of 11 FCM+/CC− versus three of 40 FCM−/CC− patients, further supporting the clinical impact of a FCM+/CC− result.[9]

Interestingly, a more detailed analysis of the results reported in the literature shows occurrence of a few CC+/FCM− discrepant cases, which typically remain unexplained.[4,21,22,31] Because of this, most studies recommend combined use of FCM and CC to increase sensitivity in diagnosing CNS disease.[9,21-23,31] In our series, only one case showed an apparently discrepant FMC−/CC+ result. However, immunocytochemical analyses performed on the cytologically positive sample, ruled out the B-cell origin of the suspicious cells, further confirming the higher negative predictive value of FCM over CC.

To the best of our knowledge, this is the first report in which a prospective multicentric study was successfully applied to the centralized study of CSF samples from a large cohort of aggressive B-NHL. Also for the first time, we successfully applied a single-tube FCM measurement of CSF cells, independently of the subtype of B-NHL and the particular phenotype of the neoplastic B-cells. For the success of the first goal, collection of CSF samples in an efficient stabilization solution[32] associated with irrelevant and controlled dilutional effects, was crucial[32] for both preventing cell loss and allowing calculation of absolute cell counts. Previous studies have shown that CSF samples are most frequently characterized by containing low cell counts which decay rapidly in their native medium,[20,33-36] if no prior preservation or fixation procedures are used.[22,32] By using Transfix, we were able to preserve leukocytes at relatively high median numbers in noninfiltrated samples, confirming that this stabilizing reagent prevents deterioration of cells in CSF samples, for hours and even days[32] (Table A1 online only). In turn, the use of a standard six-color, 11-parameter combination of MAb for systematically staining CSF samples, independently of the particular phenotype of the neoplastic B cells proved to be equal or superior to those immunophenotypic approaches where combinations of reagents adapted to the specific phenotype of malignant cells, are used.[27,37] Finally, the two-step approach here proposed, allowed for a higher sensitivity among FCM− patients, while providing a more detailed confirmatory phenotypic/molecular characterization of the aberrant/clonal B-cells initially detected, in FCM+ samples.

Table 4. Multivariate Analysis of the Value of Those Clinical and Biological Disease Characteristics Associated With CNS Involvement to Predict for CSF Infiltration by Multiparameter FCM

Clinical and Biological Parameter	Score	Cases by FCM (%)		*P*
		CNS+	CNS−	
DLBCL (n = 73)				
No PB involvement	0	57	92	.05*
PB involvement	1	43	8	
BL (n = 25)				
Normal β2-M and no NS	0	0	33	
Increased β2-M levels or NS	1	40	67	.001
Increased β2-M levels and NS	2	60	0	

Abbreviations: FCM, flow cytometry; DLBCL, diffuse large B-cell lymphoma; PB, peripheral blood; BL, Burkitt's lymphoma; β2-M, B2-microglobulin; NS, neurological symptoms.

*Note that despite the association found between PB infiltration and CSF involvement by FCM in DLBCL patients the predictive value of this parameter for CSF disease was low (sensitivity of only 43% with a rate of false positive results of 8%).

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Of note, in our series, relatively low percentages of neutrophils were detected in only 9% of the samples, providing evidence for the lack of significant levels of PB contamination in virtually all instances; this is further supported by the fact that none of the CSF+ samples containing neutrophils, showed evidence of PB infiltration by neoplastic B cells (data not shown).

On exploring the value of different disease characteristics for predicting CNS infiltration by FCM, a significant association was found between a FCM+ result and presence of neurological symptoms, extranodal and BM infiltration, and higher β2-microglobulin serum levels; as could be expected, a significantly higher frequency of CNS involvement was also found among BL versus DLBCL. In addition, three of four tFL patients had CSF infiltration in association with disease features suggesting increased risk of CNS spread,[6] although the high frequency of CSF involvement found in tFL is not clinically representative of the disease[38] and deserves further investigations.

Multivariate analysis confirmed the independent, but low, predictive value of PB involvement among DLBCL; in contrast, in BL a scoring system based on the combination of the two independent parameters for CNS disease—high serum β2-microglobulin and neurological symptoms—was highly specific for a FCM+ result, with a sensitivity of approximately 60%.

Altogether, these results support previous observations suggesting an increased risk for CNS relapse for patients displaying unique disease features such as involvement of extranodal sites, increased β2-microglobulin, and LDH serum levels as well as the phenotype of neoplastic B cells.[3,5,6,10] However, this is the first report in which an efficient algorithm is proposed for the identification of BL patients at a very high probability of CNS infiltration. Interestingly, for DLBCL patients, presence of PB involvement was the only independent parameter associated with a FCM+ CSF, but with a low predictive value. In these patients, the possibility of blood contamination of CSF samples was ruled out, due to the absence of neutrophils in CSF samples from these patients. In turn, the relatively limited value of this parameter could be due to the use of low sensitive techniques (eg, morphology) to identify PB dissemination, pointing out the need to further investigate the predictive value of PB involvement, as assessed by more sensitive approaches such as multiparameter FCM and/or polymerase chain reaction,[28,39] to predict for a FCM+ CSF result in DLBCL.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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